Ablation of the metal ion-induced endocytosis of the prion protein by disease-associated mutation of the octarepeat region

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The neurodegenerative spongiform encephalopathies, or prion diseases, are characterized by the conversion of the normal cellular form of the prion protein PrP^{C} to a pathogenic form, PrP^{Sc} [1]. There are four copies of an octarepeat PHGG(G/S)WGQ that specifically bind Cu^{2+} ions within the N-terminal half of PrP^{C} [2–4]. This has led to proposals that prion diseases may, in part, be due to abrogation of the normal cellular role of PrP^{C} in copper homeostasis [5]. Here, we show that murine PrP^{C} is rapidly endocytosed upon exposure of neuronal cells to physiologically relevant concentrations of Cu^{2+} or Zn^{2+}, but not Mn^{2+}. Deletion of the four octarepeats or mutation of the histidine residues (H68/76 dyad) in the central two repeats abolished endocytosis, indicating that the internalization of PrP^{C} is governed by metal binding to the octarepeats. Furthermore, a mutant form of PrP that contains nine additional octarepeats and is associated with familial prion disease [6] failed to undergo Cu^{2+}-mediated endocytosis. For the first time, these results provide evidence that metal ions can promote the endocytosis of a mammalian prion protein in neuronal cells and that neurodegeneration associated with some prion diseases may arise from the ablation of this function due to mutation of the octarepeat region.

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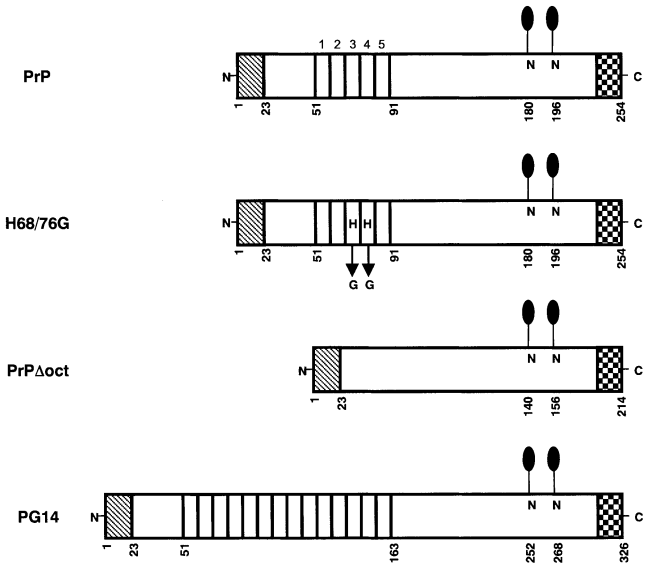
Results and discussion

Although the binding of copper to peptides corresponding to the octarepeat region of mammalian PrP has been extensively studied by biophysical methods and shown to occur primarily via the histidine residues [3, 4], an understanding of how relevant copper binding is to the function of mammalian PrP^{C} is lacking. To investigate the effect of copper binding to the octarepeats of mammalian PrP^{C}, the human neuroblastoma SH-SY5Y cell line was stably transfected with either wild-type murine PrP (wtPrP), a construct in which the octarepeat region (residues 51–90) was deleted (PrPΔoct), or one in which His68 and His76 were mutated to glycines (H68/76G) (Figure 1). All three forms of the protein were expressed as variably N-glycosylated forms on the surface of the SH-SY5Y cells (Figures 2a,b). The ability of Cu^{2+} to stimulate the endocytosis of murine wtPrP was investigated following the biotinylation of surface proteins and the incubation of the cells in serum-free medium that contained $CuSO_4$ presented as a histidine chelate. After incubation of the cells at 37°C, residual surface PrP was removed by trypsin treatment prior to the lysis of the cells. Any PrP that was endocytosed during the course of the experiment was protected from trypsin digestion. Incubation of the cells expressing wtPrP with 100 μM $CuSO_4$, which is the estimated average concentration of copper in the extracellular spaces of the brain [7], resulted in the rapid endocytosis of biotinylated murine PrP (Figure 3a). Densitometric analysis of the immunoblots indicated that 37% of the surface biotinylated PrP was endocytosed after incubation of the cells with 100 μM $CuSO_4$ for 20 min (see Figure 4g). No endocytosis was observed in the absence of $CuSO_4$ (see Figure 4a) or at concentrations of $CuSO_4$ below 100 μM (data not shown). Previously, copper has been reported to stimulate endocytosis of the chicken form of PrP [8]. However, it should be noted that there is only ~30% sequence homology between chicken and mammalian PrPs and that the region of chicken PrP that binds copper has a repeating hexapeptide motif (PHNPGY) in place of the highly conserved PHGG(G/S)WGQ octarepeat motif present in all mammalian PrPs.

In contrast to the effect of copper on wtPrP, incubation of the cells expressing PrPΔoct or H68/76G with copper did not result in the internalization of either of these forms of PrP (Figures 3b,c, respectively). Even incubation of the cells for up to 90 min with 1000 μM $CuSO_4$ failed to result in the endocytosis of PrPΔoct or H68/76G (Figures 3f,g, respectively), although wtPrP was rapidly endocytosed at this higher concentration of copper (Figure 3e). The lack of the internalization of the PrPΔoct construct clearly shows for the first time that the octarepeats are essential for the copper-stimulated endocytosis of PrP and that although Cu^{2+} may bind to other sites in the protein [9], these binding sites are not critical for this cellular response.

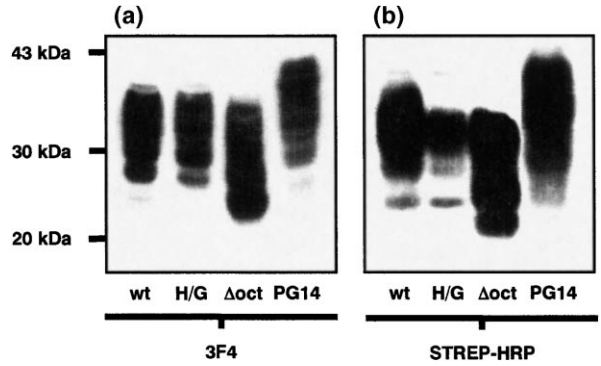
Spectroscopic studies with single- and multioctarepeat-

Figure 1



A schematic of the prion protein constructs. Wild-type murine PrP (wtPrP) contains an N-terminal 22 amino acid signal sequence (diagonally hatched box), two N-linked glycosylation sites (N180 and N196, lollipops), and a C-terminal 24 amino acid sequence (checkered box), which is replaced posttranslationally by a glycosylphosphatidylinositol moiety. The one incomplete (PQGGTWGQ, no.1) and four complete (PHGG(G/S)WGQ, nos. 2–5) octarepeats (residues 58–90) are indicated. PrPΔoct lacks all five octarepeats (residues 51–90). In H68/76G, the histidines in the central two octarepeats (nos. 3 and 4) are mutated to glycines. cDNAs encoding PrPΔoct and H68/76G were constructed by oligonucleotide-directed mutagenesis using the Stratagene Quick Change Mutagenesis Kit. The mutants generated were confirmed by DNA sequencing and subcloned into the mammalian expression vector pIRESneo (Clonetech). PG14 has an additional nine copies of the octarepeat [14, 15]. The cDNA encoding PG14 was obtained from Dr. D. A. Harris and subcloned into pIRESneo. wtPrP and all the constructs contain methionine residues substituted at L108 and V111, which allow the proteins to be recognized by the monoclonal antibody 3F4 [18].

Figure 2



Wild-type and mutant PrP constructs are expressed on the surface of the SH-SY5Y cells. SH-SY5Y cells were stably transfected with wild-type or mutant PrP cDNA constructs as described [19, 20], and pooled clones were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/ml penicillin-streptomycin. **(a)** SH-SY5Y cells stably expressing either wtPrP (wt), H68/76G (H/G), PrPΔoct (Δoct), or PG14 were washed twice with phosphate-buffered saline (PBS) and then scraped into PBS. Following centrifugation at 100 × g for 3 min, the cell pellet was resuspended in lysis buffer (10 mM Tris/HCl [pH 7.8], 0.1 M NaCl, 10 mM EDTA, 0.5% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, protease inhibitor cocktail [Sigma]) and incubated at room temperature for 30 min. The resulting lysates were clarified by centrifugation at 13000 × g for 5 min. PrP in the cell lysates was detected following SDS-PAGE and transfer to poly(vinylidene difluoride) (PVDF) membranes with antibody 3F4 [18] as described previously [19, 20]. **(b)** Cells were incubated for 30 min at 4°C in Opti-MEM containing 0.5 mg/ml Biotin-X-NHS. Cells were then washed twice at 4°C in PBS containing 50 mM glycine and lysed in lysis buffer. PrP constructs were immunoprecipitated from the cell lysate with antibody 3F4, resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with streptavidin-conjugated horseradish peroxidase.

containing peptides suggested that at a pH above 7, each octarepeat independently binds a Cu^{2+} ion [3, 10]. Since an alteration in copper-stimulated endocytosis of PrP almost certainly reflects an alteration in copper binding to the octarepeats, our data with glycosylated, glycosylphosphatidylinositol-anchored forms of mammalian PrP^C expressed in a neuronal cell line would suggest that the octarepeats do not independently bind a single Cu^{2+} ion. If this were the case, mutation of H68 and H76 would be expected to lead to a form of PrP that could still bind two Cu^{2+} ions via the intact octarepeats and therefore undergo copper-mediated endocytosis, albeit possibly at a reduced level. Rather, our data implies that PrP cooperatively binds the Cu^{2+} ions. This would be consistent with the model proposed from studies with a variety of peptide fragments that showed that at just above pH 7, the octarepeats cooperatively bind the Cu^{2+} ions through a ring-like structure composed of four metal ions and four bridging imidazolate ions [4, 9]. With such cooperative binding between the four octarepeats and the four Cu^{2+} ions, mutation of His68 and His76 would have a dramatic effect on copper binding, as evidenced by the complete block of the endocytosis in this mutant form of PrP (Figures 3c,g). The implication from these observations is that disruption of one or more octarepeats drastically compromises the endocytosis of PrP and thus perturbs the normal cellular function of the protein, possibly explaining why deletions of one or more octarepeats can give rise to prion disease [11].

Since several forms of familial prion disease are due to the insertion of extra copies of the octarepeat [6, 12, 13], we investigated the effect of additional octarepeats on the copper-mediated endocytosis of PrP, reasoning that with extra copper binding capability, such forms of the protein may be more sensitive to copper-mediated endocytosis. For this purpose, we used PG14, a construct of murine PrP that contains an additional nine octarepeats

Figure 3

Copper induces the endocytosis of wtPrP, but not the endocytosis of the mutants. SH-SY5Y cells expressing either **(a,e)** wtPrP, **(b,f)** PrPΔoct, **(c,g)** H68/76G, or **(d,h)** PG14 were surface biotinylated by incubation for 1 hr at 4°C with 0.5 mg/ml Biotin-X-NHS in Dulbecco's PBS and then rinsed three times with PBS containing 50 mM glycine. The cells were incubated with Opti-MEM at 37°C in the presence of either (a–d)100 μM $CuSO_4$ for zero or 20 min or (e–h)1000 μM $CuSO_4$ for up to 90 min, and were subsequently either lysed in lysis buffer (−) or incubated for 5 min at 37°C with trypsin/EDTA (GIBCO) prior to lysis (+). PrP constructs were immunoprecipitated with antibody 3F4, resolved by SDS-PAGE, transferred to PVDF membranes, and detected with peroxidase-conjugated streptavidin.

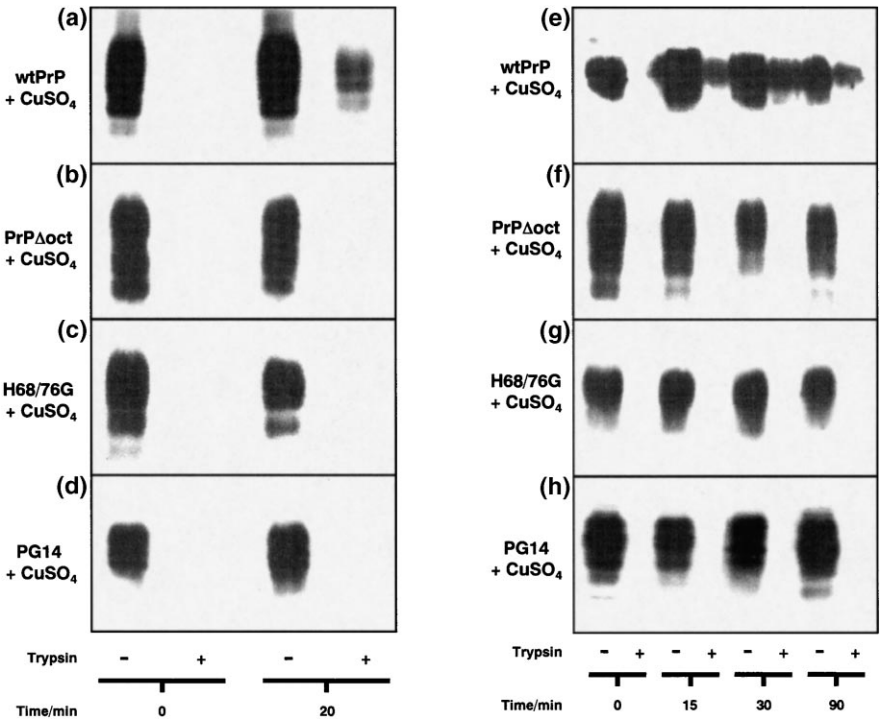
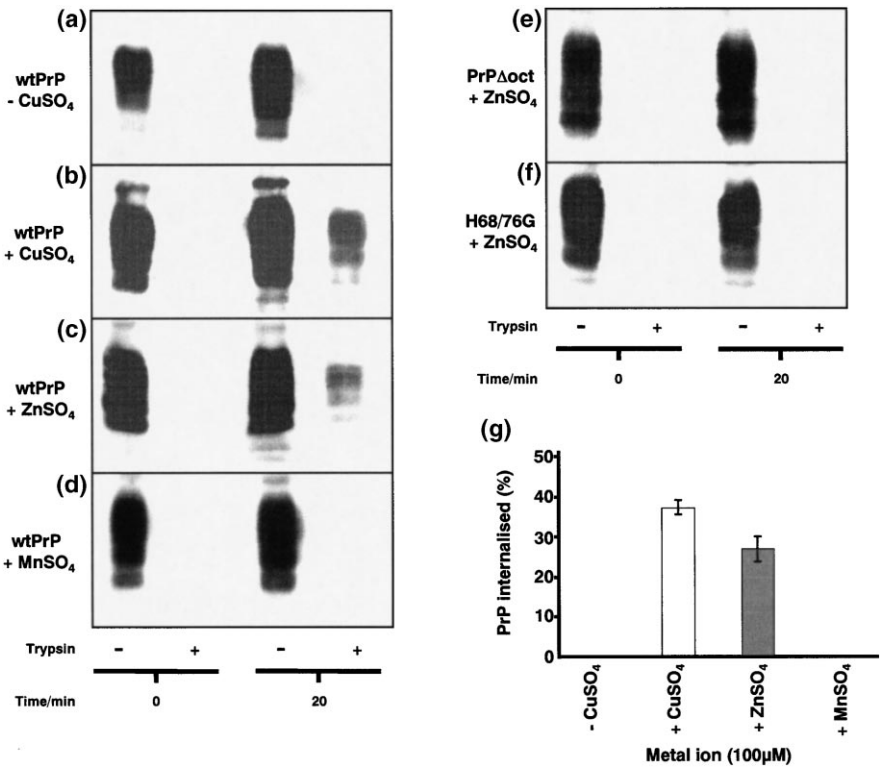


Figure 4

Zinc, but not manganese, also induces endocytosis of PrP. SH-SY5Y cells expressing either **(a–d)** wtPrP, **(e)** PrPΔoct, or **(f)** H68/76G were surface biotinylated. Following quenching with glycine, cells were incubated in Opti-MEM in the (a) absence or presence of either (b)100 μM $CuSO_4$, (c,e,f) 100μM $ZnSO_4$, or (d) 100μM $MnSO_4$ for zero or 20 min at 37°C and subsequently either lysed (−) or incubated for 5 min at 37°C with trypsin/EDTA prior to lysis (+). PrP constructs were immunoprecipitated with antibody 3F4 and detected with streptavidin-conjugated horse-radish peroxidase. The **(g)** graph shows the percentage of surface biotinylated PrP internalized by the different metal ions after 20 min at 37°C. The results are the mean (±SEM) of triplicate experiments.



(Figure 1) associated with familial human prion disease [14, 15]. This construct, like wtPrP, was expressed on the surface of the SH-SY5Y cells as a variably glycosylated protein (Figures 2a,b). Surprisingly though, when cells expressing PG14 were incubated with 100 μM or 1000 μM $CuSO_4$, there was no evidence for the endocytosis of this form of PrP (Figures 3d,h, respectively). This lack of copper-mediated endocytosis may be due to an inability of Cu^{2+} to bind to the octarepeats, possibly because of abnormal folding of this extended region of the polypeptide; or copper may be binding, but endocytosis is impaired due to the interaction of the protein with other components in the membrane [14]. Our data clearly demonstrate that additional copies of the octarepeat compromises the copper-mediated endocytosis of PrP. The effect of the octapeptide deletions and insertions might be explained by their effects on the interactions with other molecules required for PrP endocytosis. However, in the H68/76G mutant, the double point mutation is not likely to effect endocytosis by disrupting an interaction with another protein but is more likely to disrupt binding of copper to the octarepeats.

Studies using synthetic peptides and bacterially expressed forms of PrP have indicated that the octarepeats preferentially bind copper [7, 9]. We, therefore, investigated whether other divalent metal ions would promote the endocytosis of PrP. Surprisingly, $ZnSO_4$ at 100 μM, which is the estimated concentration of zinc in the extracellular fluid [16], also stimulated the endocytosis of wtPrP (Figure 4c), although to a lesser extent than CuSO4 (Figure 4g). However, it did not promote the endocytosis of PrPΔoct or H68/76G (Figures 4e,f, respectively). Although Mn^{2+} ions have been reported to bind to recombinant PrP during refolding [17], 100 μM $MnSO_4$ did not promote the endocytosis of wtPrP (Figure 4d). These data indicate that zinc, like copper, can stimulate the endocytosis of PrP by binding to the octarepeats, implying that, in vivo, PrP^C may also act as a zinc binding protein and/or transporter. Furthermore, our data suggest that studies with synthetic peptides or bacterially expressed PrP may not truly reflect the in vivo interactions between PrP^C and metal ions.

Essentially, our data implies that a function of mammalian PrP^C may be the binding/uptake of a subset of metal ions into neuronal cells. This is the first evidence that deletions or additions of octarepeats in PrP prevent the process of metal-mediated endocytosis and attribute a physiologically relevant role to this highly conserved region of mammalian PrP. The neurodegeneration seen in individuals with deletions or insertions of octarepeats in the gene encoding PrP could therefore be a result of the ablation of the copper-mediated endocytosis of PrP^C. Whether or not other disease-associated mutations in PrP perturb the copper-induced endocytosis of the protein merits further investigation.

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